Positive allosteric modulation of A_1 adenosine receptors as a novel and promising therapeutic strategy for anxiety

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ABSTRACT

Activation of A_1 adenosine receptors (ARs) has been associated with anxiolytic-like effects in different behavioral tests, but development of A_1AR agonists for therapeutic use has been hampered, most likely due to the presence of side effects. With the aim to identify a safer approach for the treatment of anxiety, we investigated, in mice, the anxiolytic-like properties of a novel A_1AR positive allosteric modulator, TRR469. Acute administration of TRR469 (0.3 – 3 mg/kg) resulted in robust anxiolytic-like effects in the elevated plus maze, the dark/light box, the open field and the marble burying tests. The magnitude of the anxiolytic action of TRR469 was comparable to that obtained with benzodiazepine diazepam (1 mg/kg). The use of the A_1AR antagonist DPCPX (3 mg/kg) suggested that the effects of TRR469 were mediated by this receptor subtype. In contrast to diazepam, the novel positive allosteric modulator did not potentiate the sedative effect of ethanol (3.5 g/kg) evaluated by the loss of righting reflex. While diazepam produced motor coordination impairment in the rotarod test, this effect being enhanced by the presence of ethanol (1.5 g/kg), TRR469 did not elicit locomotor disturbances either when administered alone or in the presence of ethanol. *In vitro*, TRR469 was able to increase the number of A_1AR recognizable by the agonist radioligand [^{3}H]-CCPA in mouse brain regions involved in emotional processes. TRR469 markedly increased the affinity of the agonist CCPA, suggesting the capability, *in vivo*, to increase the affinity of endogenous adenosine. Taken together, these findings indicate that the positive allosteric modulation of A_1AR may represent a promising approach for the treatment of anxiety-related disorders.

Keywords

Adenosine; anxiety; positive allosteric modulation; TRR469; benzodiazepines; A1 receptors

Abbreviations: AR, adenosine receptor; CCPA, 2-chloro N(6)-cyclopentyladenosine; DPCPX, 1,3-diprop-yl-8-cyclopentylxanthine; LORR, loss of righting reflex; PD 81,723, (2-amino-4,5-dimethyl-3-thienyl)-[3-(trifluromethyl)-phenyl] methanone; T62, (2-amino-4,5,6,7-tetrahydrobenzo[b]thiophen-3-yl)-(4-chlorophenyl)-methanone; TRR469, (2-Amino-4-[(4-(phenyl)piperazin-1-yl)methyl]-5-(4-fluorophenyl)thiophen-3-yl)-(4-chlorophenyl)methanone.

1. Introduction

Anxiety disorders are among the most prevalent and disabling psychiatric disorders worldwide. Selective serotonin reuptake inhibitors and benzodiazepines are the most commonly prescribed drugs for the treatment of anxiety even if their continued use has been associated with various side effects. (Bystritsky et al., 2013; Barth et al., 2016). In particular, the adverse effects of benzodiazepine include psychomotor and cognitive impairment, memory loss, sedation as well as the development of tolerance and dependence. Moreover, they are frequently implicated in polydrug intoxication in combination with other central nervous system depressants such as ethanol or opioids (Murphy et al., 2016). Accumulating evidence indicates that adenosine receptors (ARs) could be considered potential targets for innovative anxiolytic drugs. Adenosine is a neuromodulator that exerts its functions through the activation of four G-protein coupled receptors named A_1, A_{2A}, A_{2B} and A_3 ARs. In the central nervous system (CNS) adenosine is uniquely positioned to integrate inhibitory and excitatory neurotransmission mainly acting on A_1 and A_{2A} subtypes, respectively (Boison, 2008). For these reasons, one of the most promising targets for the development of new anxiolytic drugs is A_1AR, the activation of which modulates neuronal activity by blocking neurotransmitter release and reducing the firing rate (Chen et al., 2013). Recently, it has been shown that upregulating A_1ARs in forebrain neurons evokes both resilience against depressive-like behavior and antidepressant effects in a chronic depression model (Serchov et al., 2015). Different studies have highlighted that mice lacking adenosine A_1ARs display enhanced anxiety (Johansson et al., 2001; Giménez-Llort et al., 2002; Lang et al., 2003). Moreover, the

anxiogenic actions of adenosine antagonists, such as caffeine, in animals and humans have generally been attributed to the blockade of this receptor subtype (Jain et al., 1995, Florio et al., 1998, Millan, 2003). Moderate doses of caffeine, however, are reported to induce anxiolytic-like effects most likely due to the blockade of A_{2A}ARs (Cunha et al., 2008; Hughes et al., 2014). It has been reported that activation of A_1ARs mediates the anxiolytic-like effect induced by ethanol in the elevated plus maze in mice (Prediger et al., 2004). A subsequent study by the same research group, demonstrated that the A_1AR agonist 2-Chloro-N6-cyclopentyladenosine (CCPA) reduced the anxiogenic-like behavior observed during acute ethanol withdrawal in mice (Prediger et al., 2006).

Despite their promising therapeutic potential, the use of A_1AR agonists has been hampered by important side effects and poor receptor subtype selectivity (Romagnoli et al., 2010).
In particular, A_1AR activation mediates negative chronotropic and inotropic effects in the heart, catalepsy and depressant effect on locomotor activity (Kiesman et al., 2009; Chen et al., 2013). Furthermore, the clinical development of A_1AR agonists has met with limited success due to a well-defined receptor desensitization in human trials. In this regard, positive allosteric modulation has proven to represent a valuable alternative to orthosteric agonists by acting on a distinct site and potentiating the effect of the endogenous agonist (Childers et al., 2005; Gao et al., 2005). In the last decade, we have identified and characterized different series of A_1AR positive allosteric modulators, some of which represent the most potent and effective so far synthesized (Romagnoli et al., 2008, 2014). Recently, we have demonstrated the antinociceptive properties of the novel A_1AR positive allosteric modulator 2-Amino-4-[(4-(phenyl)piperazin-1-yl)methyl]-5-(4-fluorophenyl)thiophen-3-yl)-(4-chlorophenyl)methanone (TRR469) in two models of acute pain such as writhing and formalin tests and in chronic streptozotocin-induced diabetic neuropathy (Vincenzi et al., 2014).

The aim of the present study was to investigate the anxiolytic properties of TRR469 using a variety of anxiety-related tests such as elevated plus maze, open field, dark/light box and marble

burying. The ethanol interaction and potential side effects were evaluated in the loss of righting reflex and rotarod test in comparison with the reference anxiolytic diazepam. In vitro, the effect of TRR469 was analyzed on A_1AR binding parameters in mouse hippocampus, amygdala and prefrontal cortex membranes.

2. Materials and methods

2.1. Animals

Male CD1 mice (22-24 g) were obtained from Charles River (Milan, Italy) and housed five per cage in 42.5 x 26.6 x 15.5 cm polycarbonate cages. The animals were kept under standard environmental temperature and humidity-controlled conditions (22±2°C) with 12 hours light/dark cycle (light on at 7:00 AM) with food and water *ad libitum*. The mice were allowed to acclimatize to the animal facility for one week before testing. All procedures used in the present study were carried out in accordance with European Communities Council directives (2010/63/EU) and National Laws and Policies (D.L.26/2014) with the authorization from the Italian Ministry for Health. All experimental testing sessions were conducted during the light phase, between 9:00 AM and 1:00 PM. Each mouse was tested only once to ensure the novelty and avoid habituation.

2.2. Drugs

The A_1AR positive allosteric modulator TRR469 was synthesized by the Department of Pharmaceutical Sciences of the University of Ferrara (compound 4ad in Romagnoli et al., 2012) and the chemical structure is shown in Fig. 1. Diazepam, CCPA, R-N6-(phenylisopropyl)adenosine (R-PIA) and 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX) were purchased from Sigma-Aldrich (St. Louis, MO). Vehicle consisted of saline containing 5% DMSO and 5% Tween 20. Drugs were dissolved in DMSO and further diluted in vehicle. Ethanol was diluted in saline to yield doses of

1.5 and 3.5 g/kg. TRR469, diazepam or DPCPX were administered intraperitoneally in a volume of 5 µl/g body weight. Dose range of TRR469 was selected on the basis of preliminary experiments. Diazepam dose (1 mg/kg) was selected based on literature data where it showed anxiolytic-like effects in behavioral tests in mice (Micale et al., 2009). A "non-anxiogenic" dose (3 mg/kg) of the A_1AR antagonist DPCPX was selected according to previous literature (Prediger et al., 2004). Unless mentioned otherwise, drugs or their vehicle were given 15 min prior to the test.

2.3. Elevated plus maze

The elevated plus maze apparatus (Ugo Basile, Milan, Italy) consisted of a center platform (5 x 5 cm) elevated 60 cm from the floor, with attached 2 opposite open arms (35 x 5 cm) and 2 opposite closed arms (35 x 5 x 15 cm) attached and painted in non-reflective grey. The mice were placed in the center platform facing the open arm and allowed to freely explore the maze for 5 min (Lister et al., 1987). Animal behavior was recorded by means of a video camera mounted above the maze and subsequently analyzed by a researcher blinded to the experimental treatment. The number of entries into the open and closed arms and time spent in the open arms were analyzed.

2.4. Dark/light box test

The test is based on the innate aversion of rodents to brightly illuminated areas and on the spontaneous exploratory behavior in response to novel environment and light (Crawley and Goodwin, 1980). The dark/light box apparatus measured 45 x 25 cm and was divided into two chambers: a smaller dark chamber (1/3 of the total length) and a larger light chamber lit with a bright white light. A small opening door (5 x 7 cm) was located in the separator wall between the two chambers which allowed the mice to move freely between the light and dark compartments for 5 min. The mice were placed in the dark chamber and activity was recorded by means of a video camera positioned above the apparatus. The latency to enter, the time spent in the light

compartment and the total number of transitions were analyzed by a researcher blinded to the experimental treatment.

2.5. Open field test

The open field was made up of a 40 x 40 cm Plexiglas chamber with the floor divided into a grid of equally sized areas (10 x 10 cm) for visual scoring of activity by the experimenter. Rodents typically spend a significantly greater amount of time exploring the periphery of the arena, usually in contact with the walls (thigmotaxis), than the unprotected center area (Prut and Belzung, 2003). Each mouse was placed in the center of the open field arena and its behavior was recorded for 5 min. The latency to leave the central area defined by the four central squares and the time spent in the central zone were analyzed as a measure of anxiety-related behavior.

2.6. Marble burying test

The testing apparatus consisted of a polycarbonate mouse cage (30 × 20 cm) filled to a depth of 5 cm with pine wood bedding. Prior to each test, 20 glass marbles (10 mm diameter) were evenly spaced and arranged in a grid-like fashion across the surface of the bedding. Mice were placed in the apparatus and their behavior was recorded for 30 min. At the end of the test session, the mice were carefully removed from the chamber and the number of buried marbles (2/3 or more of the marble covered by bedding) was determined (Deacon, 2006).

2.7. Ethanol-induced loss of righting reflex

For the loss of righting reflex test, mice were given an intraperitoneal injection of 3.5 g/kg ethanol. When the animals lost the righting reflex, they were placed in a plastic cage, and the time was recorded by an observer blinded to drug treatments. Mice were judged to have regained their righting reflex when they could right themselves three times within 1 min after being placed on

their backs.

Vehicle, diazepam (1 mg/kg) or TRR469 (1,3 and 10 mg/kg) were intraperitoneally administered 15 min before ethanol.

2.8. Rotarod test

Changes in motor performance were measured using a fixed speed (12 rpm) rotarod (Ugo Basile, Milan, Italy). Mice received two training trials on two separate days prior to testing for acclimatization. They were trained to remain on the rotarod, and those that did not remain on the bar for two consecutive periods of 300 sec were eliminated (Vincenzi et al., 2013). On the experimental day, the time that the mice remained on the rotating bar (cut-off 300 s) was recorded 15 min after the intraperitoneal injection of different concentrations (1-10 mg/kg) of diazepam or TRR469. To study the interaction with ethanol, the mice were injected with vehicle, diazepam or TRR469 after the administration of a sub-effective dose of ethanol (1.5 g/kg). 15 min after drug injection, their ability to remain on the rotarod was then tested and their latency to fall was recorded.

2.9. Membrane preparation

For membrane preparation mouse hippocampus, amygdala and prefrontal cortex were rapidly removed, resuspended in 50 mM Tris-HCl (pH 7.4) and homogenized by means of a Polytron. The homogenate was then centrifuged for 20 min at 40000 *g* and the resulting pellet resuspended and incubated for 30 min in 50 mM Tris-HCl (pH 7.4) in the presence of 3 IU/ml of adenosine deaminase. The suspension was then centrifuged again for 20 min at 40000 *g* and the membrane pellet homogenized in 50 mM Tris-HCl (pH 7.4) and used for binding experiments. The protein concentration was determined according to a Bio-Rad method with bovine albumin as standard reference.

2.10. [^{3}H]-CCPA and [^{3}H]-DPCPX binding experiments

Saturation binding experiments of [^{3}H]-CCPA (0.05–20 nM) in mouse hippocampus, amygdala and prefrontal cortex membranes were performed in triplicate at 25°C for 90 min in 50 mM Tris-HCl, pH 7.4, in the absence and in the presence of TRR469. Non-specific binding was defined with 1 μM R-PIA (Vincenzi et al., 2014). Saturation binding experiments of [^{3}H]-1,3-diprop-yl-8-cyclopentylxanthine ([^{3}H]-DPCPX, 0.05-20 nM) in mouse hippocampus, amygdala and prefrontal cortex membranes were performed in triplicate at 25°C for 90 min in 50 mM Tris-HCl, pH 7.4. Competition experiments were carried out by incubating 1 nM [^{3}H]-DPCPX with membrane suspension (80 μg of protein/100 μl) and different concentrations of CCPA (0.01 nM-1 μM) at 25°C for 90 min in 50 mM Tris-HCl, pH 7.4. Non-specific binding was defined as binding in the presence of 1 μM DPCPX (Vincenzi et al., 2014).

Bound and free radioactivity were separated by filtering the assay mixture through Whatman GF/B glass fibre filters using a Brandel cell harvester (Brandel Instruments, Unterföhring, Germany). The filter bound radioactivity was counted using a Packard Tri Carb 2810 TR scintillation counter (Perkin Elmer, Boston, MA, USA).

2.11. Statistical and data analysis

All statistical analyses were performed by using the statistical package software Graph Pad Prism version 6.0 (GraphPad Software, Inc., La Jolla, CA). For comparisons between multiple groups, one-way ANOVA with Newman-Keuls multiple comparison test was used. For antagonist studies and interaction with ethanol, two-way ANOVA followed by Bonferroni post hoc test was used. Significance was set at $p < 0.05$. Data are presented as mean and standard error of the mean. Student's t-tests were used for direct comparisons between K_D, Bmax and Ki values. Inhibitory binding constant values, Ki, were calculated from the IC_{50} according to the Cheng & Prusoff

equation Ki= $IC_{50}/(1+[C^*]/K_D^*)$, where [C*] is the concentration of the radioligand and K_D^* its dissociation constant (Vincenzi et al., 2014).

3. Results

3.1. TRR469 exerts anxiolytic-like effects in the elevated plus maze

The A_1AR positive allosteric modulator TRR469 showed anxiolytic effects similar to diazepam in the elevated plus maze (Fig. 2A and B). One-way analysis of variance revealed a main effect of treatment evaluating the time spent in the open arms ($F_{4,55} = 7.6$, $p < 0.0001$) and the percentage of entries in the open arms ($F_{4,55} = 5.3$, $p = 0.0011$). Post hoc analysis revealed a significant increase in the exploration of the open arms induced by diazepam (1 mg/kg) or TRR469 at 1 mg/kg ($p < 0.01$) and 3 mg/kg ($p < 0.001$) in comparison to vehicle-injected mice (Fig. 2A). Diazepam (1 mg/kg) and TRR469 (1 and 3 mg/kg) showed similar significant increases ($p < 0.01$) in the percentage of entries into the open arms (Fig. 2B). Pretreatment with DPCPX (3 mg/kg), an A_1AR antagonist, completely abrogated the anxiolytic-like effect of TRR469 (1 mg/kg) in the elevated plus maze (Fig. 2C and D). In particular, for the time spent in the opens arms, two-way analysis of variance revealed a significant effect of TRR469 treatment ($F_{1,44} = 10.54$, $p = 0.0022$), a significant effect of DPCPX treatment ($F_{1,44} = 7.63$, $p = 0.0083$), and a significant interaction between TRR469 and DPXPC treatments ($F_{1,44} = 4.85$, $p = 0.033$). Bonferroni post hoc analysis confirmed a significant effect of TRR469 (1 mg/kg) compared to vehicle ($p < 0.01$) and a significant effect of TRR469 (1 mg/kg) + DPCPX (3 mg/kg) compared to TRR469 alone ($p < 0.01$). For the entries into the open arms, two-way ANOVA revealed a significant effect of TRR469 treatment ($F_{1,44} = 9.01$, $p = 0.0044$), a significant effect of DPCPX treatment ($F_{1,44} = 8.02$, $p = 0.0069$), and a significant interaction between TRR469 and DPXPC treatments ($F_{1,44} = 5.31$, $p = 0.026$). Bonferroni post hoc analysis showed a significant effect of TRR469 (1 mg/kg) compared to

vehicle ($p < 0.01$) and a significant effect of TRR469 (1 mg/kg) + DPCPX (3 mg/kg) compared to TRR469 alone ($p < 0.01$).

3.2. Anxiolytic effects of TRR469 in the dark/light box test

In the dark/light box test, both diazepam and TRR469 determined an increase in the time spent in the light compartment during a 5-min session ($F_{4,55} = 10.2$, $p < 0.0001$) and a decrease in the latency to leave the dark compartment ($F_{4,55} = 8.3$, $p < 0.0001$). Newman-Keuls post hoc test revealed a significant increase in the time spent in the light chamber induced by diazepam (1 mg/kg, $p < 0.001$) or TRR469 at the doses of 0.3 mg/kg ($p < 0.05$), 1 mg/kg ($p < 0.01$) and 3 mg/kg ($p < 0.01$) compared to vehicle (Fig. 3A). As shown in Fig. 3B, the latency to enter the light compartment was significantly reduced by diazepam (1 mg/kg, $p < 0.001$) or TRR469 at 1 mg/kg ($p < 0.01$) and 3 mg/kg ($p < 0.001$). The increase in the time spent in the light compartment and reduction in the latency to leave the dark compartment induced by 1 mg/kg TRR469, was completely blocked by the A_1AR antagonist DPCPX (Fig. 3C and D). For the time spent in the light compartment, two-way ANOVA revealed a significant effect of TRR469 treatment ($F_{1,44} = 18.89$, $p < 0.0001$), a significant effect of DPCPX treatment ($F_{1,44} = 6.48$, $p = 0.0145$), and a significant interaction between TRR469 and DPXPC treatments ($F_{1,44} = 4.86$, $p = 0.033$). Bonferroni post hoc analysis confirmed a significant effect of TRR469 (1 mg/kg) compared to vehicle ($p < 0.001$) and a significant effect of TRR469 (1 mg/kg) + DPCPX (3 mg/kg) compared to TRR469 alone ($p < 0.01$). For the latency to leave the dark compartment, two-way ANOVA revealed a significant effect of TRR469 treatment ($F_{1,44} = 5.36$, $p = 0.0253$), a significant effect of DPCPX treatment ($F_{1,44} = 11.04$, $p = 0.0018$), and a significant interaction between TRR469 and DPXPC treatments ($F_{1,44} = 11.21$, $p = 0.0017$). Bonferroni post hoc analysis showed a significant effect of TRR469 (1 mg/kg) compared to vehicle ($p < 0.01$) and a significant effect of TRR469 (1 mg/kg) + DPCPX (3 mg/kg) compared to TRR469 alone ($p < 0.01$).

3.3. Assessment of behavioral selectivity

To evaluate the effect of the different treatments on general locomotor activity, the frequency of closed arm entries in the elevated plus maze and the number of total transitions in the dark/light box tests were evaluated. TRR469, as well as diazepam or DPCPX, did not alter the number of closed arm entries during the 5-min session of the elevated plus maze ($F_{6,77} = 0.4$, $p = 0.85$, Fig. 4A). Analogously, TRR469, diazepam or DPCPX treatments did not modify the total number of transitions between the dark and the light compartments in the dark/light box test respect to vehicle-injected mice ($F_{6,77} = 0.2$, $p = 0.97$, Fig. 4B).

3.4. TRR469 reduces anxiety-related behaviors without affecting locomotor activity in the open field test

Diazepam and TRR469 significantly increased the time spent in the center and the latency to leave the center of the open field arena compared to vehicle ($F_{4,55} = 4.7$, $p = 0.0023$ and $F_{4,55} = 5.6$, $p = 0.0008$, respectively). In particular, mice spent more time in the central area of the open field when treated with diazepam (1 mg/kg, $p < 0.05$) or TRR469 at doses of 1 mg/kg ($p < 0.05$) and 3 mg/kg ($p < 0.01$) compared to the vehicle-injected mice (Fig. 5A). Diazepam-treated mice (1 mg/kg) exhibited significantly longer latencies to leave the inner zone of the open field ($p < 0.01$), than did the vehicle-treated mice (Fig. 5B). Similar results were obtained with injection of TRR469 1 mg/kg ($p < 0.05$) and 3 mg/kg ($p < 0.01$).

The number of squares crossed in the open field was evaluated to exclude a possible influence of anxiolytic doses of diazepam or TRR469 on spontaneous locomotor activity. As depicted in Fig. 5C, the number of squares crossed by mice treated with diazepam or TRR469 did not significantly differ from vehicle-injected mice ($F_{4,55} = 0.3$, $p = 0.86$).

3.5. TRR469 inhibits marble-burying behavior in mice

As expected, vehicle-treated mice buried a high number of marbles (17.5 ± 0.7) during the 30-min session. One-way ANOVA revealed a significant effect of treatment with diazepam or TRR469 in the number of marbles buried ($F_{4,45} = 6.9$, $p = 0.0002$, Fig. 5D). Post hoc analysis confirmed that a significantly smaller number of marbles was buried after treatment with diazepam 1 mg/kg (5.5 ± 2.3, $p < 0.01$) as well as with TRR469 injected at doses of 1 mg/kg (6.2 ± 2.2, $p < 0.01$) or 3 mg/kg (4.7 ± 2.1, $p < 0.01$).

3.6. Diazepam, but not TRR469, potentiated ethanol-induced loss of righting reflex and motor impairment

For the ethanol interaction study, an acute dose of ethanol (3.5 g/kg), capable of inducing a mean loss of righting reflex duration of 29 min, was used (Fig. 6A). No treatment was able to induce a loss of righting reflex in the absence of ethanol. Evaluating diazepam (1 mg/kg) effect, two-way ANOVA revealed a significant effect of diazepam treatment ($F_{1,28} = 24.31$, $p < 0.0001$), a significant effect of ethanol treatment ($F_{1,28} = 89.73$, $p < 0.0001$), and a significant interaction between diazepam and ethanol ($F_{1,28} = 24.31$, $p < 0.0001$). Bonferroni post hoc test confirmed a significant effect of diazepam compared to vehicle + ethanol ($p < 0.001$). For TRR469, two-way ANOVA revealed no effect of treatment or interaction between TRR469 and ethanol ($F < 1.0$).

To examine interactions with ethanol in the rotarod test, mice were pretreated with a sub-effective dose of ethanol (1.5 g/kg) prior to the administration of diazepam or TRR469 (Fig. 6B). Two-way ANOVA revealed a significant effect of diazepam treatment ($F_{3,56} = 130.0$, $p < 0.0001$), a significant effect of ethanol treatment ($F_{1,56} = 217.5$, $p < 0.0001$),and a significant interaction between diazepam and ethanol ($F_{3,56} = 29.04$, $p < 0.0001$). Bonferroni post hoc analysis showed that diazepam significantly decreased the latency to fall when administered at 3 mg/kg ($p < 0.05$) or 10 mg/kg ($p < 0.001$) compared to vehicle. The combined treatment with increasing doses of diazepam

(1, 3 and 10 mg/kg) and ethanol produced a marked reduction in the time spent on the rotarod, which was significantly lower than diazepam alone ($p < 0.001$). In contrast, TRR469 failed to produce any locomotor disturbances at all the tested doses (1, 3 and 10 mg/kg, Fig. 6B). Combined treatment with increasing doses of TRR469 (1, 3 and 10 mg/kg) and ethanol did not affected rotarod performance as revealed by two-way ANOVA that showed no effect of TRR469 treatment and no interaction between TRR469 and ethanol (*F<1.0).*

3.7. TRR469 enhances binding characteristics of the A_1AR agonist CCPA in different mouse brain regions

Saturation binding experiments of the radioligand [^{3}H]-CCPA performed in membranes from mouse hippocampus, amygdala and prefrontal cortex in the absence or in the presence of the positive allosteric modulator TRR469 are reported in Fig. 7A, B and C, respectively. The presence of the positive allosteric modulator determined a significant increase in [^{3}H]-CCPA affinity (K_D) in all the substrates investigated. Moreover, TRR469 (10 μM) induced a Bmax value increase of 2.8-fold in hippocampus ($p < 0.001$), 2.1-fold in amygdala ($p < 0.001$) and 2.2-fold in prefrontal cortex ($p < 0.001$) membranes (Table 1). Receptor density (Bmax values) obtained in the presence of TRR469 reflected the shift of the receptor from the inactive to the active state therefore increasing the number of receptor sites recognizable by the agonist radioligand [^{3}H]-CCPA. The total density of A_1ARs was then evaluated with the antagonist radioligand [^{3}H]-DPCPX, that does not discriminate between active and inactive state of the receptors (Table 1). As expected, the presence of TRR469 did not affect the K_D nor the Bmax value of the antagonist radioligand [^{3}H]-DPCPX. One of the great advantages of allosteric ligands is the possibility to increase the affinity of endogenous ligands by exploiting their physiological effects instead of activating the receptors with an exogenous orthosteric drug. To assess this potential effect, we evaluated the affinity of the adenosine analogue CCPA in the presence of the novel allosteric enhancer TRR469. In [^{3}H]-

DPCPX competition binding experiments performed in hippocampus membranes, the presence of TRR469 10 μM induced a 16.5-fold increase in CCPA affinity ($p < 0.001$: Fig. 7D). Similar results were obtained in mouse amygdala or prefrontal cortex membranes (Fig. 7E and F; Table 1).

4. Discussion

The pharmacological treatment of anxiety-related disorders usually involves the prescription of benzodiazepines or serotonin reuptake inhibitors. The use of benzodiazepines, however, is often associated with abuse, tolerance and dependence issues as well as various side effects such as sedation and cognitive impairment (Buffett-Jerrott and Stewart, 2002). Despite their mild side effects, serotonin reuptake inhibitors are characterized by a delayed onset of anxiolytic effects. A need, therefore, exists for novel, fast-acting anxiolytic agents characterized by fewer side effects.

Previous works highlighted A_1ARs as a potential target for the development of novel anxiolytic drugs (Jain et al., 1995; Florio et al., 1998; Johansson et al., 2001; Giménez-Llort et al., 2002; Prediger et al., 2004; Prediger et al., 2006). Despite this evidence, various side effects, above all cardiovascular effects and motor impairments, associated with direct A_1AR activation have hampered the clinical use of A_1 agonists (Elzein et al., 2008; Kiesman et al., 2009). In this context, allosteric receptor modulation represents an attractive concept in drug targeting offering important potential advantages over conventional orthosteric ligands (Wang et al., 2009). In particular, since positive allosteric modulators enhance the function of receptors activated by endogenous agonist, they are expected to have a much lower side effect potential than orthosteric agonists, a low propensity for receptor desensitization and a high selectivity for a given receptor subtype (Urwyler, 2011). We have previously reported the synthesis and pharmacological characterization of the novel positive allosteric modulator TRR469 (Romagnoli et al., 2012). In comparison to the currently available A_1 positive allosteric modulator reference compounds, T62 or PD 81,723, the novel

compound TRR469 showed an allosteric potency that was at least 10-fold higher than that obtained for the two reference allosteric enhancers (Vincenzi et al., 2014).

In the present study, we examined the potential anxiolytic-like action of the positive allosteric modulator TRR469 in various tests of anxiety in mice. When evaluated in a classical test, such as the elevated plus maze, TRR469 exhibited robust anxiolytic-like effects comparable to those of benzodiazepine diazepam, which was used as a positive control. A similar effect in the elevated plus maze had previously been observed with the use of low doses of A_1 agonists, even if this effect was lost at higher doses, most likely due to the motor depressant action of direct A_1ARs activation (Jain et al., 1995; Prediger et al., 2004). To confirm the anxiolytic properties of TRR469, the allosteric modulator was then tested in the dark/light box test, that is based on the innate aversion of rodents to brightly illuminated areas. It is well reported that benzodiazepine anxiolytics increase the time spent by the animal in the light compartment and decrease the latency to leave the dark zone (Chaouloff et al., 1997; Li et al., 2009). In this test, the magnitude of the effect of TRR469 was comparable to that of diazepam, confirming the potential anxiolytic-like action of this compound. Pretreatment with the A_1AR antagonist DPCPX abolished the anxiolytic-like effect of TRR469 in the elevated plus maze and dark/light box test, most likely preventing the binding of endogenous adenosine to this receptor subtype. In the elevated plus maze and dark/light box tests, TRR469 did not alter the frequency of the closed arm entries and the number of transitions, respectively. The lack of effect on these parameters, related to general locomotor activity, demonstrated the behavioral selectivity of the A_1AR positive allosteric modulator TRR469.

In the open field test, the time spent in the central area and the latency to leave the center were chosen as parameters of anxiety-related behavior, while the number of squares crossed was considered a measure of general locomotor activity. TRR469 exhibited an anxiolytic effect similar to diazepam without affecting the locomotor activity of the mice. To complete the characterization of the anxiolytic properties of TRR469, we used the marble-burying test as a tool for assessing both

anxiety-like and repetitive-like behaviors in mice. Similarly to diazepam, TRR469 reduced the number of marbles buried and although this test alone should not be considered predictive of an anxiolytic action (Thomas et al., 2009), numerous anxiolytic drugs have proven to be effective in this test (Nicolas et al., 2006).

Even if benzodiazepines are generally considered safe, their interaction with ethanol is a major concern as it produces a marked impairment of psychomotor performance in both humans and animals. We have shown that, in contrast to diazepam, the A_1AR allosteric modulator TRR469 did not enhance ethanol-induced loss of righting reflex, even when tested at a 10-fold higher dose than the anxiolytic dose. These results also suggest the lack of sedative side effects by TRR469. Deficits in motor coordination is another known adverse effect of benzodiazepines. In the present study, we have confirmed that not only does diazepam produce motor coordination impairments *per se*, it also has even greater effects when administered in combination with sub-effective doses of ethanol. In contrast, TRR469 did not impair rotarod performance either *per se*, or in the presence of ethanol. In a previous work, we demonstrated that the direct activation of A_1ARs with the agonist CCPA resulted in pronounced motor coordination impairment and cataleptic effects (Vincenzi et al., 2014), thus suggesting the potential advantages of positive allosteric modulators.

To shed some light on the mechanism of action of the anxiolytic-like properties of TRR469, we investigated *in vitro* its effect on agonist binding parameters in hippocampus, amygdala and prefrontal cortex, brain regions intimately involved in stress, anxiety and emotional responses. The increase in the number of A_1ARs recognizable by the agonist radioligand [^{3}H]-CCPA obtained in the presence of TRR469 is consistent with the known effect of positive allosteric modulators of G-coupled receptor to shift the receptor state in the active form (Christopoulos and Kenakin, 2002). These results are in agreement with those found for the prototypic A_1AR allosteric modulator PD 81,723 (Kollias-Baker et al., 1997). In particular, it has been shown that some allosteric modulators are able to modulate the coupling of the receptor with the G protein and this can result in an

enhancement of maximal orthosteric ligand binding capacity (Bmax) by increasing the number of binding sites recognizable by the agonist radioligands (Christopoulos and Kenakin, 2002). This is also confirmed by the lack of TRR469 modulation of the antagonist radioligand [^{3}H]-DPCPX binding that does not discriminate the active and the inactive (or G protein coupling and uncoupling) state of the receptor. One of the great advantages of positive allosteric modulators is their ability to increase endogenous agonist affinity, enhancing the activation of the receptor in a more physiological way. In the present study we have shown that TRR469 was able to increase the affinity of the adenosine analogue CCPA in hippocampus, amygdala and prefrontal cortex membranes with an increment of 14, 17 or 32 fold, respectively.

Despite in the present work, we have highlighted some of the potential of A_1AR positive allosteric modulators as anxiolytic agents, some questions remains to be addressed. In particular, future works will be focused on the evaluation of the effects of chronic administration of TRR469 to verify if the anxiolytic-like properties are maintained over time. Furthermore, issues linked to desensitization and cardiorespiratory side effects, typically associated with the use of A_1AR agonists, are still to be elucidated.

In conclusion, the current study demonstrates for the first time the potential anxiolytic-like action of A_1AR positive allosteric modulators. In particular, TRR469 displayed an anxiolytic profile similar to diazepam in various anxiety tests in mice. Furthermore, in contrast to diazepam, it did not show interaction with ethanol to induce sedation and motor impairment. Taken together, these data provide compelling evidence to support the positive allosteric modulation of A_1ARs as a new interesting pharmacological strategy for the treatment of anxiety-related disorders.

Disclosure/Conflicts of interest

The authors have no financial interests to disclose.

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Table 1

Binding parameters of the A_1AR agonist CCPA and the antagonist DPCPX in the absence or in the presence of TRR469

	[^{3}H]-CCPA saturation binding assays		[^{3}H]-DPCPX saturation binding assays		[^{3}H]-DPCPX competition binding assays
	K_D (nM)	**Bmax (fmol/mg protein)**	**K_D (nM)**	**Bmax (fmol/mg protein)**	**CCPA Ki (nM)**
Hippocampus					
Control	2.08±0.19	145±12	0.45±0.03	429±37	8.59±0.56
+TRR469	0.66±0.05***	406±34***	0.47±0.03	436±35	0.52±0.03***
Amygdala					
Control	2.51±0.16	92±8	0.65±0.05	223±15	8.86±0.68
+TRR469	0.92±0.11***	196±13***	0.62±0.06	228±17	0.63±0.05***
Prefrontal cortex					
Control	1.80±0.14	119±8	0.41±0.03	322±21	9.95±0.74
+TRR469	0.65±0.04***	264±16***	0.43±0.04	316±24	0.31±0.02***

Data are expressed as mean ± SEM of four independent experiments performed in duplicate.
$^{***}p < 0.001$ vs control, Student's t-test.

Figure legends

Fig. 1. Chemical structure of the A_1AR positive allosteric modulator TRR469 (2-Amino-4-[(4-(phenyl)piperazin-1-yl)methyl]-5-(4-fluorophenyl)thiophen-3-yl}-(4-chlorophenyl)methanone).

Fig. 2. Anxiolytic-like effects of TRR469 in the elevated plus maze. Effect of diazepam (Diaz, 1 mg/kg) or TRR469 (0.3 – 3 mg/kg) on the time spent in the open arms (A) and on the percentage of entries into the open arms (B) in the elevated plus maze during a 5-min session. $^{**}p < 0.01$ vs vehicle, Newman-Keuls multiple comparison test; $^{***}p < 0.001$ vs vehicle, Newman-Keuls multiple comparison test. Pretreatment with the A_1 antagonist DPCPX (3 mg/kg) abrogated the effect of TRR469 (1 mg/kg) on the time spent in the open arms (C) and on the percentage of entries into the open arms (D) in the elevated plus maze during a 5-min session. TRR469 or diazepam were intraperitoneally injected 15 min before the test, while DPCPX was intraperitoneally injected 15 min before TRR469 (n = 12 per treatment condition). $^{**}p < 0.01$ vs vehicle + vehicle, Bonferroni post hoc test; $^{\#\#}p < 0.01$ vs vehicle + TRR469 1 mg/kg, Bonferroni post hoc test.

Fig. 3. Anxiolytic-like effects of TRR469 in the dark/light box test. (A) Effect of diazepam (Diaz, 1 mg/kg) or TRR469 (0.3 – 3 mg/kg) on the time spent in the light compartments (A) and on the latency to enter the light chamber (B) during a 5-min session. $^{*}p < 0.05$ vs vehicle, Newman-Keuls multiple comparison test; $^{**}p < 0.01$ vs vehicle, Newman-Keuls multiple comparison test; $^{***}p < 0.001$ vs vehicle, Newman-Keuls multiple comparison test. Pretreatment with the A_1 antagonist DPCPX (3 mg/kg) blocked the effect of TRR469 (1 mg/kg) on the time spent in the light compartments (C) and on the latency to enter the light chamber (D) in the dark/light box test during a 5-min session. TRR469 or diazepam were intraperitoneally injected 15 min before the test, while DPCPX was intraperitoneally injected 15 min before TRR469 (n = 12 per treatment condition). $^{**}p$

< 0.01 vs vehicle + vehicle, Bonferroni post hoc test; $^{***}p < 0.001$ vs vehicle + vehicle, Bonferroni post hoc test; $^{\#\#}p < 0.01$ vs vehicle + TRR469 1 mg/kg, Bonferroni post hoc test.

Fig. 4. Assessment of general locomotor activity. (A) Effect of diazepam (Diaz, 1 mg/kg), TRR469 (0.3 – 3 mg/kg) or DPXPC (3 mg/kg) on the number of entries into the closed arms in the elevated plus maze during a 5-min session. (B) Effect of diazepam (Diaz, 1 mg/kg), TRR469 (0.3 – 3 mg/kg) or DPXPC (3 mg/kg) on the total number of transitions between the dark and the light compartment in the dark/light box test during a 5-min session. TRR469 or diazepam were intraperitoneally injected 15 min before the test, while DPCPX was intraperitoneally injected 15 min before TRR469 (n = 12 per treatment condition).

Fig. 5. Effect of diazepam (Diaz, 1 mg/kg) and TRR469 (0.3 – 3 mg/kg) on the time spent in the central area (A), on the latency to leave the central area (B) and on the number of squares crossed (C) in the open field test during a 5-min session. Drugs were intraperitoneally injected 15 min before the test (n = 12 per treatment condition).). $^{*}p < 0.05$ vs vehicle, Newman-Keuls multiple comparison test; $^{**}p < 0.01$ vs vehicle, Newman-Keuls multiple comparison test. (D) Effect of diazepam (Diaz, 1 mg/kg) and TRR469 (0.3 – 3 mg/kg) on the number of buried marbles during a 30-min session. Drugs were intraperitoneally injected 15 min before the test (n = 12 per treatment condition). $^{**}p < 0.01$ vs vehicle, Newman-Keuls multiple comparison test.

Fig. 6. TRR469 have a better side effect profile than diazepam. (A) Effect of diazepam (Diaz, 1 mg/kg) and TRR469 (1 – 10 mg/kg) on ethanol-induced loss of righting reflex. Ethanol (3.5 g/kg) was intraperitoneally injected 15 min after diazepam or TRR469 administration (n = 8 per treatment condition). $^{***}p < 0.001$ vs vehicle + ethanol, Bonferroni post hoc test. (B) Effect of diazepam (Diaz, 1 – 10 mg/kg) and TRR469 (1 – 10 mg/kg) in the absence (white bars) or in the presence

(black bars) of a sub-effective dose of ethanol (1.5 g/kg) in the rotarod test. Drugs were administered 15 min before the evaluation on the rotarod (n = 8 per treatment group). $^{\S}p < 0.05$ vs vehicle + vehicle, Bonferroni post hoc test; $^{***}p < 0.001$ vs vehicle + ethanol, Bonferroni post hoc test; $^{\#\#\#}p < 0.001$ vs vehicle + diazepam at the same dose, Bonferroni post hoc test.

Fig. 7. Effect of TRR469 on agonist and antagonist binding parameters. Saturation curves of specific [^{3}H]-CCPA binding in mouse hippocampus (A), amygdala (B) or prefrontal cortex (C) membranes in the absence (filled circles) or in the presence (open circles) of TRR469 10 µM. [^{3}H]-DPCPX competition binding curves of CCPA in mouse hippocampus (D), amygdala (E) or prefrontal cortex (F) membranes in the absence (filled circles) or in the presence (open circles) of TRR469 10 µM. Data are expressed as mean ± SEM of four independent experiments performed in duplicate.

Figure 1

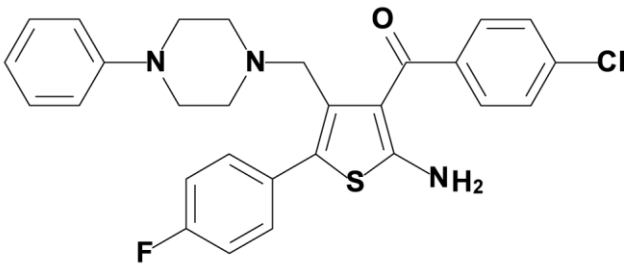


Figure 2

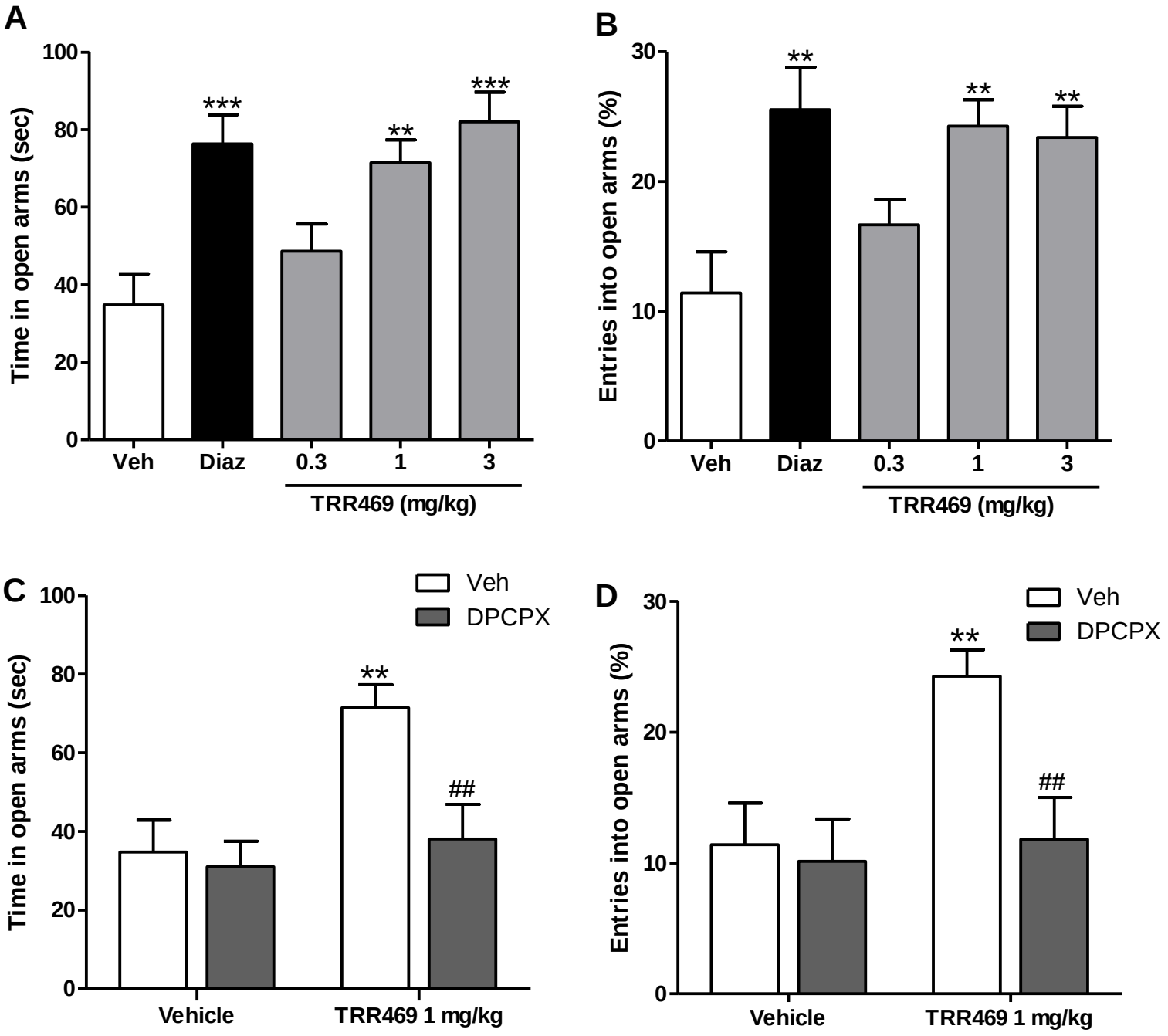

A
Time in open arms (sec)
100
80
60
40
20
0
***
**
***
Veh
Diaz
0.3
1
3
TRR469 (mg/kg)
B
Entries into open arms (%)
30
20
10
0
**
**
**
Veh
Diaz
0.3
1
3
TRR469 (mg/kg)
C
Time in open arms (sec)
100
80
60
40
20
0
Veh
DPCPX
**
##
Vehicle
TRR469 1 mg/kg
D
Entries into open arms (%)
30
20
10
0
Veh
DPCPX
**
##
Vehicle
TRR469 1 mg/kg

Figure 3

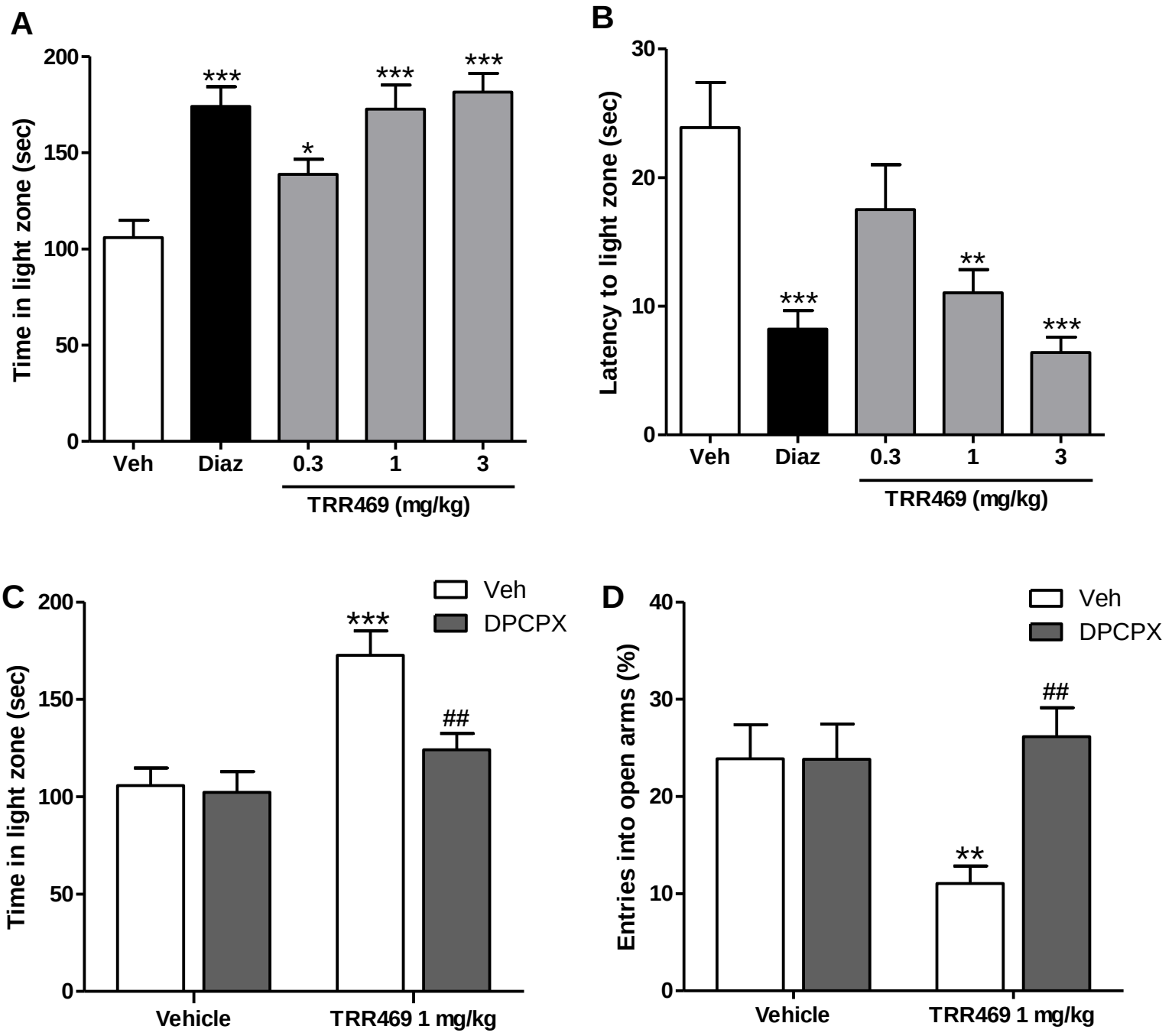

A
Time in light zone (sec)
200
150
100
50
0
***
***
*
***
Veh
Diaz
0.3
1
3
TRR469 (mg/kg)
B
Latency to light zone (sec)
30
20
10
0
***
**
***
Veh
Diaz
0.3
1
3
TRR469 (mg/kg)
C
Time in light zone (sec)
200
150
100
50
0
Veh
DPCPX
***
##
Vehicle
TRR469 1 mg/kg
D
Entries into open arms (%)
40
30
20
10
0
Veh
DPCPX
**
##
Vehicle
TRR469 1 mg/kg

Figure 4

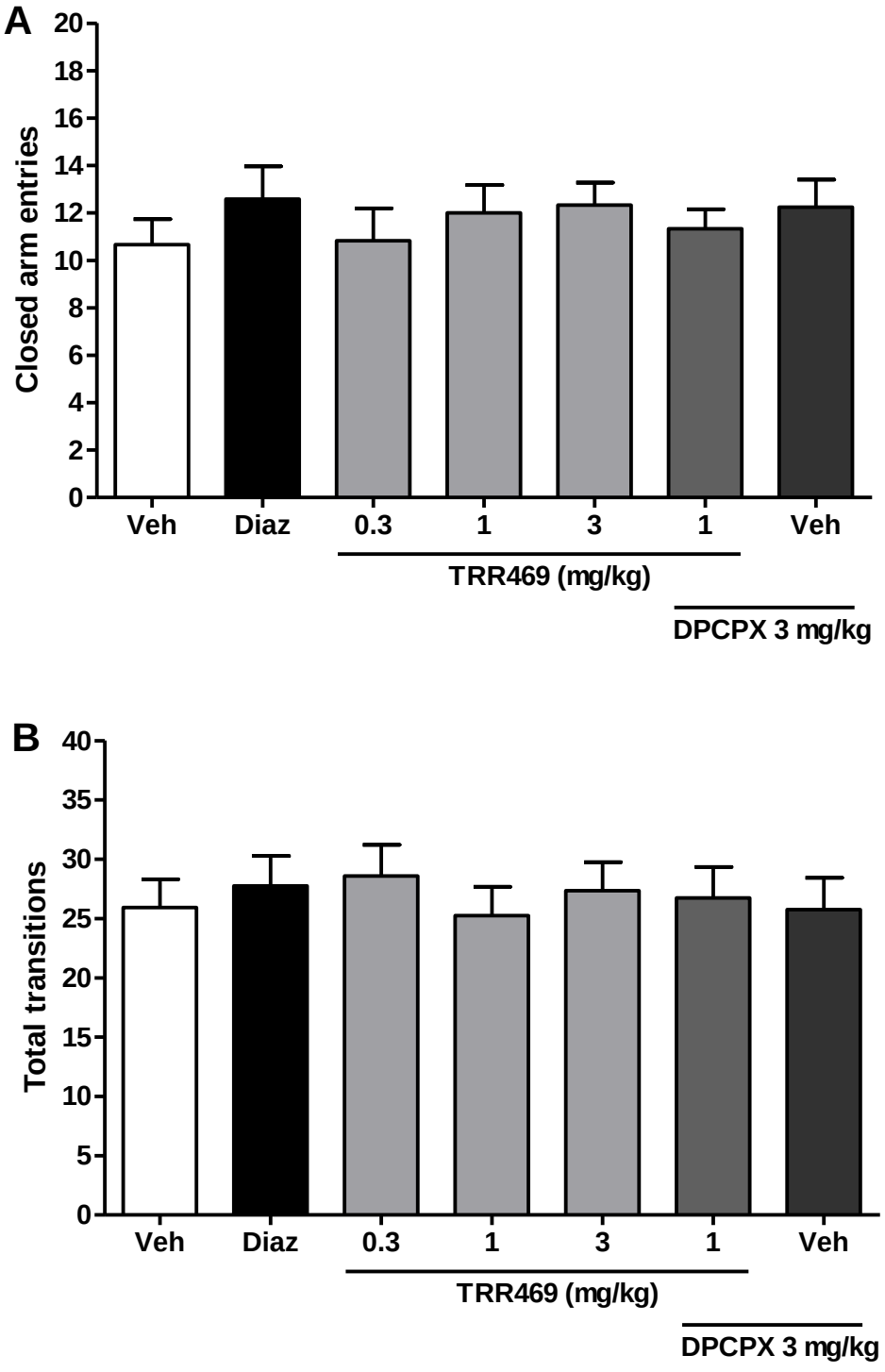

A
Closed arm entries
20
18
16
14
12
10
8
6
4
2
0
Veh
Diaz
0.3
1
3
1
Veh
TRR469 (mg/kg)
DPCPX 3 mg/kg
B
Total transitions
40
35
30
25
20
15
10
5
0
Veh
Diaz
0.3
1
3
1
Veh
TRR469 (mg/kg)
DPCPX 3 mg/kg

Figure 5

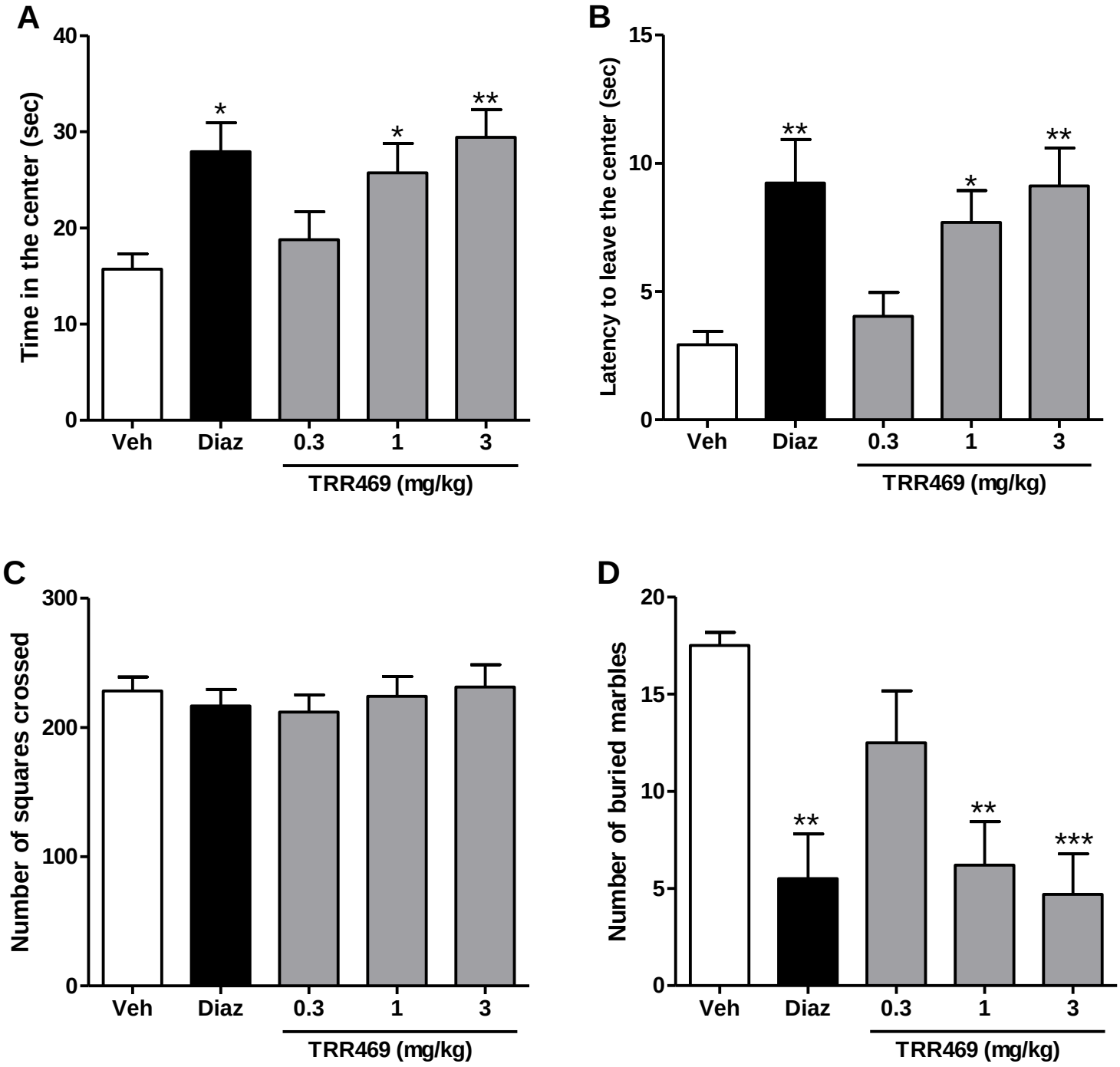

A
Time in the center (sec)
40
30
20
10
0
*
*
**
Veh
Diaz
0.3
1
3
TRR469 (mg/kg)
B
Latency to leave the center (sec)
15
10
5
0
**
*
**
Veh
Diaz
0.3
1
3
TRR469 (mg/kg)
C
Number of squares crossed
300
200
100
0
Veh
Diaz
0.3
1
3
TRR469 (mg/kg)
D
Number of buried marbles
20
15
10
5
0
**
**
***
Veh
Diaz
0.3
1
3
TRR469 (mg/kg)

Figure 6

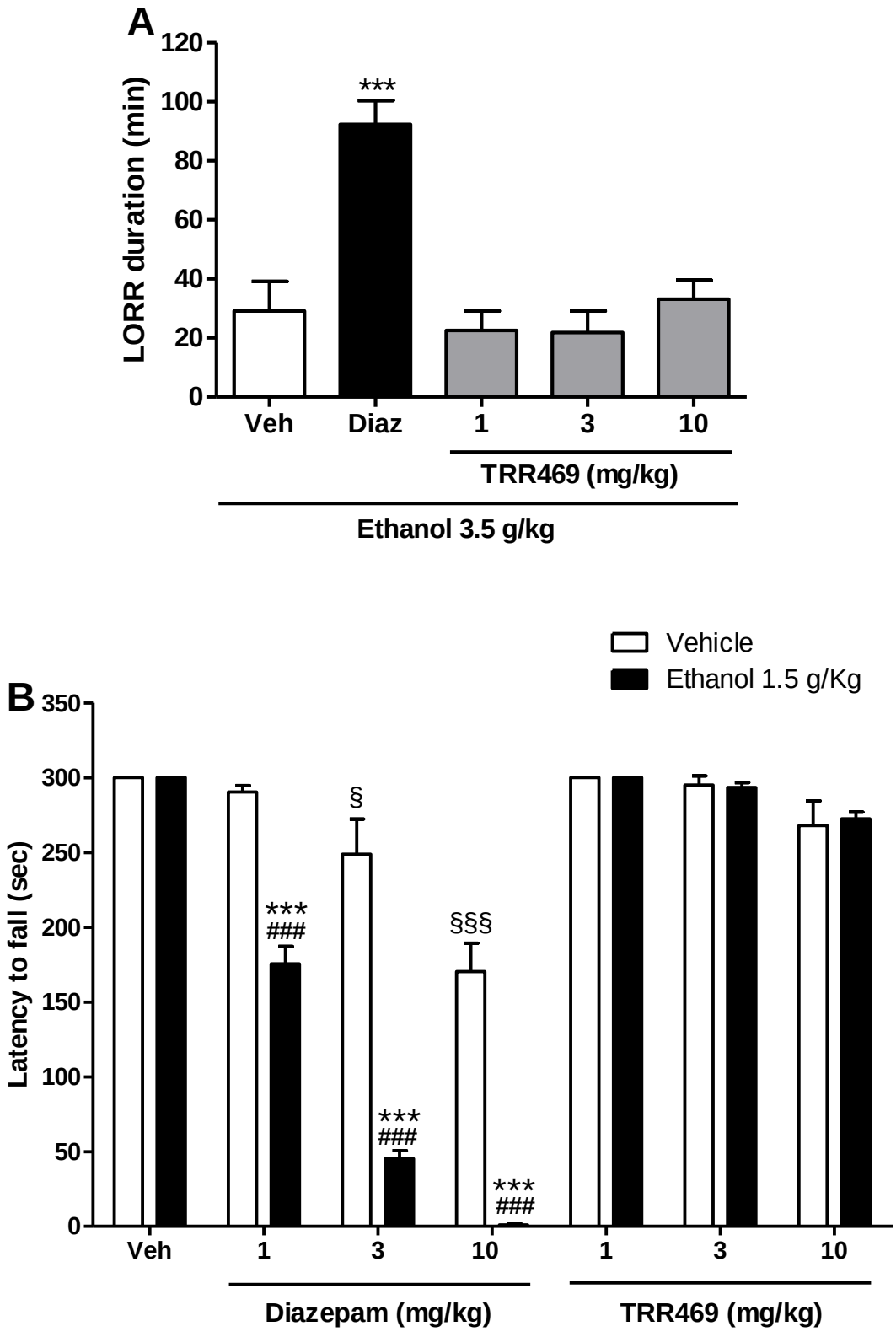
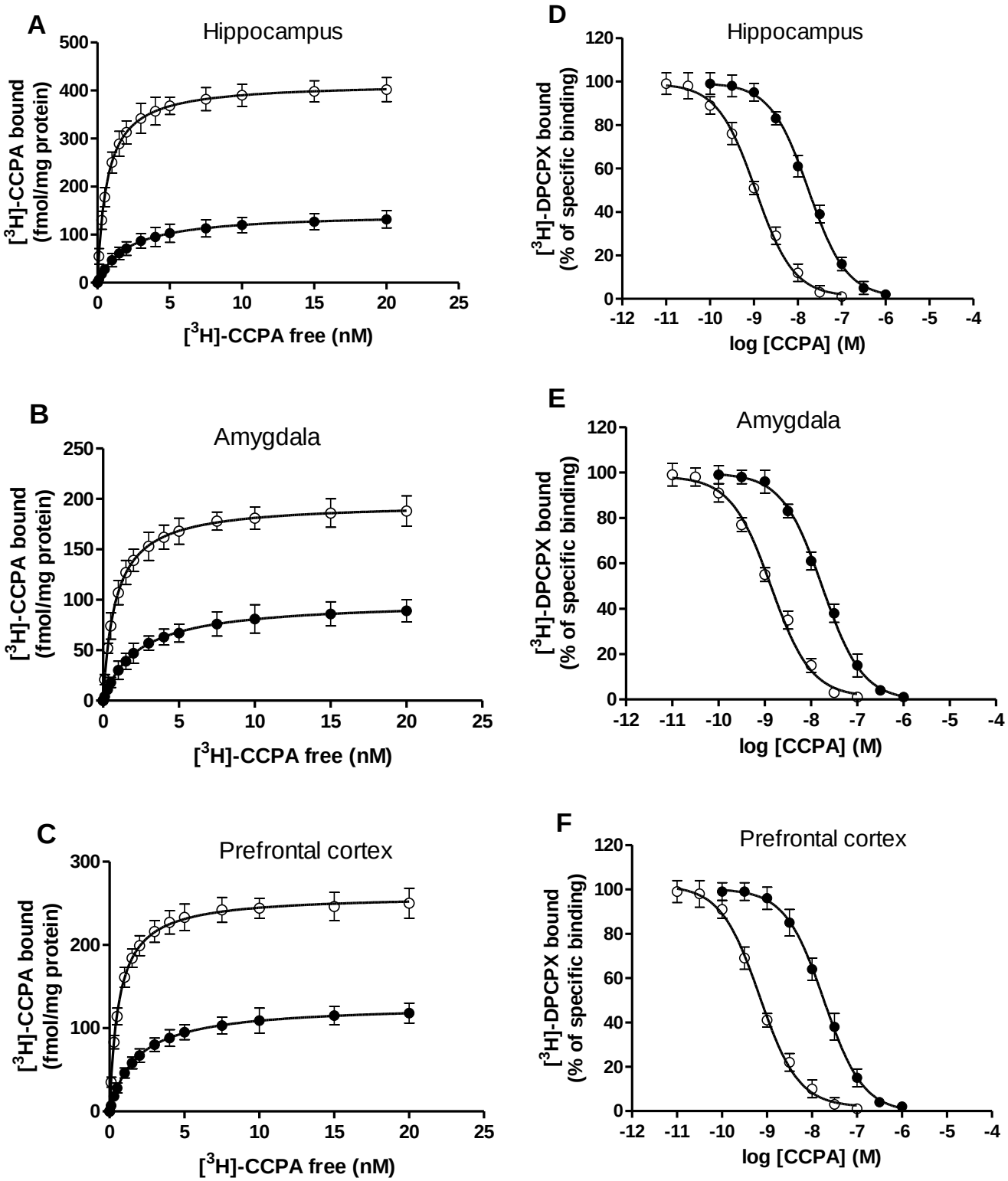

A
LORR duration (min)
120
100
80
60
40
20
0
***
Veh
Diaz
1
3
10
TRR469 (mg/kg)
Ethanol 3.5 g/kg
Vehicle
Ethanol 1.5 g/Kg
B
Latency to fall (sec)
350
300
250
200
150
100
50
0
***
###
§
§§§
***
###
***
###
Veh
1
3
10
1
3
10
Diazepam (mg/kg)
TRR469 (mg/kg)

Figure 7


A
Hippocampus
[3H]-CCPA bound (fmol/mg protein)
[3H]-CCPA free (nM)
B
Amygdala
[3H]-CCPA bound (fmol/mg protein)
[3H]-CCPA free (nM)
C
Prefrontal cortex
[3H]-CCPA bound (fmol/mg protein)
[3H]-CCPA free (nM)
D
Hippocampus
[3H]-DPCPX bound (% of specific binding)
log [CCPA] (M)
E
Amygdala
[3H]-DPCPX bound (% of specific binding)
log [CCPA] (M)
F
Prefrontal cortex
[3H]-DPCPX bound (% of specific binding)
log [CCPA] (M)